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Factors

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13. ABSTRACT (Maximum 200) Anchorage-independent growth is an important characteristic of tumor cells and the mechanisms which regulate anchorage-independent growth are not well understood. It is known that there are ECM proteins in tumor spheroids, but their function remains unclear. We have previously demonstrated that FN can promote anchorage-independent growth of SP1 cells. We now show that FN and BMA5, a blocking antibody to the $\alpha_5\beta_1$ integrin, can inhibit cell death in the absence of adhesion. Soluble FN could increase tyrosine phosphorylation of SK-LC6 cells. FAK was weakly phosphorylated under anchorage-independent conditions. We demonstrated that FN, HGF, or TGF- β can also reduce cell death by approximately 50%. When added in combination, FN and HGF or FN and TGF- β demonstrated an additive effect on cell survival. Wortmannin, an inhibitor of PI 3-kinase had very little effect on the survival induced by FN, HGF, or TGF- β individually but strongly inhibited the additive effect of ECM and growth factors on cell survival. SP1 cell clones expressing a dominant negative p85 subunit of PI 3-kinase also lost the additive effect of FN and HGF on cell survival. These results show that ECM and growth factors play an important role and act cooperatively in anchorage-independent cell survival and growth.				
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Ron Saulnier Aug 22nd/96
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INTRODUCTION

Mortality in breast cancer is primarily due to metastasis, the dissemination of tumour cells from the primary tumour to distant sites. Once in distant organs, the carcinoma cells grow in a tight spherical nodule, disturb the normal histology and eventually the function of the organ. A property of metastatic cells is that they have acquired the ability to grow anchorage-independently. They have lost the requirement for cell adhesion and spreading to proliferate as required by their non-malignant cell counterpart. During malignant transformation, the microenvironment of the cells is altered. These changes include upregulation or downregulation of ECM proteins as well as the corresponding integrin receptors (1,2). ECM proteins regulate many cellular functions such as cell adhesion, shape, migration and differentiation (3). A number of ECM proteins including fibronectin, laminin, collagen, and proteoglycan have been demonstrated in tumour spheroids however the function of these ECM proteins in tumour spheroids is not known (4,5).

Integrins are transmembrane heterodimers composed of an α and a β subunit non-covalently bound together. They mediate adhesion to the ECM and some cell-cell interactions (6-8), and are involved in a number of biological processes such as cell adhesion and spreading, migration, invasion and matrix remodelling (9,10).

Although there is a general reduction of integrin receptors on transformed cells, the pattern of expression is variable in each tumour and may vary considerably within a single tumour (1). The functions of β_1 integrins are being investigated, but there still remain many questions as to the role of individual integrin receptors in the various stages of tumour progression.

In CHO cells, over-expression of a transfected α_5 integrin gene inhibits anchorage-independent growth and tumorigenicity (11), thus the amount of α_5 expressed is critical.

Overexpression is growth inhibitory in some cell types, whereas other cells require low levels of $\alpha_5\beta_1$ integrin for proliferation in response to growth factors (12). This apparent paradox may be resolved with the recent finding that expression of $\alpha_5\beta_1$ integrin without ligation induces pathways leading to decreased cellular proliferation while ligation reverses this signal and induces cell proliferation (13). Pena *et al.* have shown that canine non-metastatic malignant mammary tumours express $\alpha_5\beta_1$ integrin, while expression on metastatic malignant tumours was reduced, but there were some tumours which still express considerable amounts of $\alpha_5\beta_1$ (14).

Integrin cytoplasmic domains are very small, containing only 25-50 amino acids. None of the integrins described contain kinase domains or enzymatic activity, however it is now well established that integrin receptors are coupled to intracellular signaling pathways via tyrosine kinases (reviewed in 15, 16). The early events after integrin ligation are aggregation of integrins and the cytoskeletal proteins paxillin, talin, tensin, vinculin, and α -actinin to focal adhesion contacts (17).

A number of proteins including pp125^{FAK} (18), paxillin (19), pp60^{src} (20), MAP kinase, (21) and other known proteins (22,23) have been demonstrated to be tyrosine phosphorylated after integrin ligation. A number of signaling molecules are also used by growth factor receptors, suggesting a cooperative or competitive interaction between ECM and growth factor receptors. McNamee *et al.* have shown that PIP₂ content was increased after adhesion to ECM and have suggested a cooperative interaction between adhesion and growth factor responsiveness (24). A number of reports have shown that ECM and growth factors are required for cell survival of adherent cells however little has been done to understand the mechanisms which regulate survival of transformed cells capable of anchorage-independent growth. Adhesion mediated survival through integrins has been linked to the upregulation of

Bcl-2 (25). PI 3-kinase is also important in growth factor mediated survival of PC12 cells (26). These results, primarily performed on adherent cells, indicate that specific integrin molecules generate intracellular signals which regulate different cell functions including survival. However, very little is known about whether specific ECM-integrin interactions can regulate growth factor responsiveness and anchorage-independent growth. We are using a murine mammary carcinoma cell line, SP1 which can grow anchorage-independently similar to *in vivo* tumour nodules to examine ECM interactions that regulates growth factor responsiveness of these cells.

Our preliminary results suggest that both ECM (FN) and growth factors (HGF and TGF- β) are required to promote anchorage-independent growth. Therefore, we need to look at the cooperative interactions between adhesion-mediated signals and growth factor signals.

In this report we show that both ECM proteins and growth factors are able to promote survival of SP1 cells under anchorage-independent conditions. We also observed an additive increase in survival which is PI 3-kinase dependent when a combination of ECM and growth factors were added to anchorage-independent SP1 cells.

Results

Our main goal is to better understand the mechanisms by which extracellular matrix and growth factors can promote anchorage-independent growth of a murine mammary carcinoma cell line. Our objectives are to investigate: (1)(a) the role of fibronectin and the $\alpha_5\beta_1$ integrin in SP1 cell survival as well as (b) the phosphorylation status of FAK under anchorage-independent conditions; (2) the role of HGF and TGF- β in SP1 mediated cell survival; (3) and the role of PI 3-kinase in SP1 cell survival and anchorage-independent growth.

Ia. $\alpha_5\beta_1$ integrin mediates SP1 cell survival under anchorage-independent conditions

We have previously shown that fibronectin can increase colony growth of SP1 cells and that the integrin $\alpha_5\beta_1$ is the predominant fibronectin receptor on the cell surface of these cells (27). Addition of soluble bovine plasma fibronectin to SP1 cells maintained under anchorage-independent conditions on agar resulted in a 50% decrease in the number of cells dying over a 24 h period while collagen and laminin had no significant effect on cell survival (Figure 1). Last year we demonstrated that BMA5 (supplied by B. Chan) was a blocking antibody to the murine α_5 integrin subunit. Addition of the BMA5 antibody to SP1 cells maintained under anchorage-independent conditions also reduced cell death while control IgG or a non-blocking antibody to the α_5 subunit had no effect (Figure 2). Interestingly, addition of a secondary rabbit-anti rat IgG, which should promote cross-linking resulted in an increase in cell death (see discussion). Addition of the GoH3 antibody which is a blocking antibody to the $\alpha_6\beta_1$ integrin had no effect on survival (data not shown).

TGF- β is known to increase fibronectin synthesis in a number of cell lines as well as to induce surface expression of a number of integrins (27,28). We have previously shown that TGF- β stimulates anchorage-independent growth of SP1 cells in agar (29). We now

investigate whether TGF- β can alter the adhesive potential of SP1 cells to extracellular matrix. SP1 cells pretreated with TGF- β under adherent conditions, for 24 h, showed an increase in adhesiveness to fibronectin, vitronectin and collagen types I and IV (Figure 3a). SP1 cells do not adhere significantly to laminin without, or with TGF- β treatment. When SP1 cells were pretreated with TGF- β , for 24 h, while maintained under anchorage-independent conditions there was an increase in adhesiveness to collagen type I and collagen type IV but not to fibronectin, vitronectin or laminin (Figure 3b). These results suggest that TGF- β may enhance colony growth of SP1 cells by altering ECM-integrin interactions at the cell surface. The effect of TGF- β on FN and collagen synthesis and integrin expression in SP1 cells maintained under adherent and anchorage-independent conditions is currently being investigated.

1b. FAK is poorly tyrosine phosphorylated under anchorage-independent conditions.

As a first step in determining the signal transduction pathways involved in fibronectin-dependent colony growth, we looked at the tyrosine phosphorylation pattern of SK-LC6 cells. SK-LC6 cells are a lung carcinoma cell line with similar properties to SP1 cells with respect to their adhesive and anchorage-independent properties (data not shown). This cell line also has a much lower baseline level of phosphorylated proteins than SP1 cell line making them more suitable for phosphorylation studies. Adhesion of SK-LC6 cells to fibronectin increased tyrosine phosphorylation of a number of proteins at the 100-110 KDa and 60-70 KDa range (Figure 4). Addition of fibronectin and to a lesser extent, laminin, but not collagen, to SK-LC6 in suspension also results in increased tyrosine phosphorylation of a number of proteins at the 100-110 KDa and 60-70 KDa range (Figure 4).

Adhesion of many cell lines to ECM proteins results in the phosphorylation of FAK, an

important component in a number of signal transduction pathways(30). We also found that adhesion of SP1 cells to fibronectin caused tyrosine phosphorylation of FAK and this phosphorylation was increased in the presence of HGF, as reported by others (31). In contrast, soluble fibronectin, HGF or fibronectin + HGF together were unable to induce FAK phosphorylation above baseline level in suspended SP1 cells (Figure 5).

II. HGF and TGF- β promote survival of SP1 cells.

We have previously shown that HGF and TGF- β can increase colony formation of SP1 cells in agar (29). We now show that HGF and TGF- β can also promote survival of SP1 cells in suspension in a concentration-dependent manner. HGF at concentrations between 5-30 ng/ml can promote survival while concentrations of TGF- β between 0.1-0.5 ng/ml are sufficient to promote survival. Concentrations of growth factor higher than 30 ng/ml for HGF and 0.5 ng/ml (for TGF- β) resulted in a decrease in cell survival. In contrast, the higher concentrations of fibronectin did not increase cell death (Figure 6).

III. The role of PI 3-kinase in fibronectin and HGF mediated cell survival.

HGF and TGF- β could inhibit cell death by approximately 50%, as did fibronectin, however when added in combination, either HGF and FN or TGF- β and FN resulted in an additive effect which was comparable to the survival obtained with 7% FBS (Figure 7).

In pursuit of a mechanism for HGF-mediated cell survival, we investigated whether PI 3-kinase is involved, since PI 3-kinase activity was shown to be required in HGF-mediated proliferation of SP1 cells on plastic (32). We used two approaches to determine whether PI 3-kinase is involved in survival of SP1 cells under anchorage-independent conditions. First, Wortmannin, an inhibitor of PI 3-kinase, could increase cell death in all groups including 7%

FBS. However, the additive effect of both FN + HGF on cell survival was strongly inhibited by wortmannin, resulting in a survival similar to HGF or fibronectin alone which were only slightly reduced as compared to controls (Figure 7).

Second, we used SP1 cells transfected with a dominant negative mutant of the p85 subunit of PI 3-kinase, designated $\Delta p85$. The C23 clone which expresses high levels of $\Delta p85$, and shows reduced PI 3-kinase activity (30), showed no additive effect on survival when HGF and FN were added together. The increased cell survival in response to HGF or FN alone was only slightly affected (Figure 8). In contrast, another transfected clone, C22, which does not express $\Delta p85$, retained an additive effect of HGF and FN on SP1 cell survival (Figure 8). The clone C23, expressing $\Delta p85$ was also less efficient than the control and transfectant C22 cells in growing anchorage-independently in soft-agar (data not shown). These results suggests that PI 3-kinase is required for FN + HGF mediated survival under anchorage-independent conditions.

Under adherent conditions PI 3-kinase showed increased tyrosine phosphorylation in response to HGF but very little in response to PLL or FN alone. Under anchorage-independent conditions PI 3-kinase showed a low level of tyrosine phosphorylation, which was increased slightly in the presence of FN, HGF and a combination of both HGF and FN with the greatest increase being in the HGF treated group (Figure 9). Protein loading controls demonstrate less proteins in the FN + HGF group therefore densitometric analysis may reveal greater PI 3-kinase activity in this group. Preliminary results looking at PI 3-kinase activity of SP1 cells under anchorage-independent conditions showed that PI 3-kinase activity was increased in cell lysates of cells treated with FN, HGF, and FN + HGF compared to untreated cells (Figure 10).

Goals and subgoals for the next year

We have three main goals for the final year.

I. Based on the results we had obtained with the SP1 cell clones in the first year, we proposed to transfect the Cl-24-L clone with the α_2 integrin subunit to determine whether over-expression improves colony forming ability. This experiment is currently being carried out in collaboration with B. Chan (University of Western Ontario, Canada). We will also transfect a human breast carcinoma cell line, EL-E, which lacks the α_5 subunit, with the full length α_5 integrin cDNA, as well as with the α_5 cDNA containing deletions in the cytoplasmic domain. These experiments will determine whether α_2 and α_5 directly affect survival and growth under anchorage-independent conditions.

II. We have not yet determined whether SP1 cell death in suspension is occurring via an apoptotic mechanism. We will look at the expression of Bcl-2 during fibronectin or HGF-mediated survival of SP1 cells using immunoprecipitation and western blotting. We will also asses DNA fragmentation using the apoptag staining procedure.

III. We will investigate the mechanisms by which TGF- β and HGF can promote anchorage-independent growth and survival of SP1 cells and the involvement of PI 3-kinase and other cytoplasmic regulators (eg. Ras and Src) in this process.

Conclusions and Discussion

We have previously demonstrated that fibronectin and growth factors (HGF and TGF- β) are required for anchorage-independent growth of SP1 cells. In this report we have demonstrated that soluble fibronectin and the BMA5 antibody can promote cell survival under anchorage-independent conditions. In addition, soluble fibronectin stimulates tyrosine phosphorylation of specific proteins in SK-LC6 cells. FAK was poorly phosphorylated in response to fibronectin under anchorage-independent conditions. These results suggest that integrins are involved in SP1 cell survival under AI conditions by FAK independent mechanisms. We also demonstrated that fibronectin, HGF or TGF- β could promote cell survival in a concentration dependent manner. Fibronectin in solution can promote cell survival and colony growth, however FAK was poorly phosphorylated under these conditions suggesting that other signaling molecules are involved. In other experiments HGF does increase the phosphorylation of FAK under adherent conditions, but also increases spreading of SP1 cells within the same time period.

Addition of the secondary Rabbit anti-rat IgG Ab causes capping and endocytosis of the receptor and generation of a signal through the $\alpha_5\beta_1$ integrin. Our results show that addition of the rabbit anti-rat IgG with the BMA5 causes an increase in cell death as opposed to the expected increase in survival which is seen with BMA5 alone. There are two possible explanations for this result (1) The addition of the primary and secondary antibody cause rapid capping and endocytosis of the receptor resulting in continuous depletion of the receptor from the cell surface, thus no continuous signal. Varner et al (1995) have shown that unligated $\alpha_5\beta_1$ generates a negative signal while ligated receptor generates a positive signal (13) hence rapid depletion of the receptor from the cell surface may mimic unligated receptor. (2) Another possibility is that there is a large proliferation signal generated as seen

with growth factors (Figure 6) under anchorage-independent conditions which causes the cells to die in the absence of adhesion.

Our results demonstrating that TGF- β increases the adhesiveness of SP1 cells to collagen under anchorage-independent conditions supports last years observations that the C1-12-H clone which grows efficiently in agar, expressed higher levels of $\alpha_2\beta_1$ and that collagen promoted colony growth of these cells. Together these results support a role for collagen and the $\alpha_2\beta_1$ receptor in anchorage-independent growth of SP1 cells. The results from Figure 3 also demonstrate that adhesive signals affect the response of SP1 cells to TGF- β providing more evidence to support a cooperative role between growth factors and extracellular matrix.

There are two possible mechanisms by which TGF- β could be promoting cell survival and anchorage-independent growth of SP1 cells; (1) direct signaling through TGF- β receptors and (2) upregulating expression of extracellular matrix and integrins. We have not conclusively shown whether direct signaling is contributing to this phenomenon, however it has been shown that TGF- β can inhibit nucleosomal fragmentation following loss of adhesion in normal human keratinocytes (33). We have shown that TGF- β upregulates adhesion to ECM under adherent conditions and especially to collagen under anchorage-independent conditions. We are currently investigating whether it upregulates the integrin expression on SP1 cells. It is possible that both mechanisms are involved. Direct signaling may be involved in promoting cell survival in early events while the upregulation of adhesion is more important for colony formation at a later time.

We have shown that SP1 cells can respond to ECM proteins and growth factors under anchorage-independent conditions as do adherent cells. We have previously demonstrated that PI 3-kinase is involved in HGF mediated proliferation of adherent SP1 cells (32). When FN + HGF or FN + TGF- β were added, there was an additive increase in survival compared to

FN or growth factor alone. The increase in survival could be inhibited by wortmannin or a dominant negative mutant of p85 subunit of PI 3-kinase suggesting that the additive effect is PI 3-kinase dependent. Together, these results support a cooperative interaction between FN (via $\alpha_5\beta_1$) and HGF (via Met receptor) in the regulation of survival and growth of SP1 cells. We now demonstrate that PI 3-kinase is involved in HGF-mediated cell survival in the presence of fibronectin under anchorage-independent conditions. Although many effector molecules have been shown to affect cell survival, this study is the first report that HGF can stimulate this effect and suggests that PI 3-kinase is an important regulatory molecule in this process. These results, together with our future studies will provide us with a better understanding of why normal cells are unable to survive and grow in the presence of extracellular matrix and growth factors under anchorage-independent conditions and the signaling pathways involved in permitting transformed cells to grow anchorage-independently. This information will greatly improve our understanding of malignant cells and lead to the development of better therapies.

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Figure legends

Figure 1. Survival assay with SP1 cells in the presence of ECM proteins. SP1 cells were harvested and seeded (20,000/dish) on agar coated dishes (60 x 15mm) in 1.5 ml RPMI supplement with 0.5mg/ml BSA and 1% FBS. ECM proteins were added at a concentration of 10 μ g/ml. After 24 h, the cells were harvested and stained with acridine orange (4 μ g/ml) and ethidium bromide (4 μ g/ml). At least 100 cells in each group were counted and the number of dead (red staining nucleus) and live (green staining nucleus) cells was assessed using a fluorescence microscope. The experiment was repeated 4 times and the mean \pm SD calculated. The results of the 1% FBS are the control group.

Figure 2. Survival assay in the presence of BMA5 antibody. The survival assay was performed as described in Figure 1. BMA5, a monoclonal blocking antibody against the α 5 integrin subunit provided as a supernatant by B. Chan, was added at 20% v/v. Rabbit anti-mouse IgG and non-blocking rat anti- α 5 mAb (Pharmingen) were added at 30 μ g/ml at the start of the assay. The results obtained with 1% FBS are the control group. The experiment was repeated 4 times and the mean \pm SD calculated. The results of the 1% FBS are the control group.

Figure 3. Adhesion assay showing the effect of TGF- β on SP1 cell adhesion. SP1 cells were cultured under adherent (panel A) or non-adherent conditions (panel B) in the presence of 2% FBS and in the absence or presence of 0.5 ng/ml TGF- β for 24 h. The cells were harvested and seeded (30,000 cells per well) in 96-well plates precoated with ECM proteins (10 μ g/ml). The cells were allowed to adhere for 45 min at 37°C. Unattached cells were washed off with PBS and the remaining cells were fixed in 3.7% paraformaldehyde and stained with

0.1% toluidine blue. Quantitation was performed using an ELISA plate reader at 570 nm.

The experiment was performed in triplicate and the results expressed as mean \pm SD.

Figure 4. Western blot on lysate of SK-LC6 cells treated with ECM proteins. SK-LC6 cells were prestraved for 24 h in RPMI 1640, harvested and allowed to adhere to fibronectin-coated plates or incubated in 10 ml tubes in the presence or absence of 40 μ g/ml fibronectin, collagen or laminin for 1 h at 37°C. The cells were lysed in RIPA lysis buffer and the lysate resolved by 8.0% SDS-PAGE under reducing conditions. The proteins were transferred to nitrocellulose and probed with anti-phosphotyrosine antibody and visualize by ECL.

Figure 5. Western blot of FAK phosphorylation under adherent and anchorage-independent conditions. SP1 cells were prestraved in RPMI 1640 for 24 h and allowed to adhere to fibronectin-coated plates or incubated in 10 ml tubes with FN (40 μ g/ml) or HGF (20 ng/ml) for 30 min at 37°C. The cells were lysed in 1% NP-40 lysis buffer and the total protein concentration standardized in each group. FAK was immunoprecipitated with anti-FAK mAb (UBI). The immunoprecipitates were washed and resolved by 8.0% SDS-PAGE. The proteins were transferred to nitrocellulose and blotted with anti-phosphotyrosine antibody then visualized by ECL.

Figure 6. Survival assay showing the effect of fibronectin, HGF and TGF- β on SP1 cell survival. The survival assay was performed as described in Figure 1. SP1 cells (20,000 per dish) were seeded in 1.5 ml RPMI 1640 with 1% FBS on agar coated plates (35 x 10mm) for 24 h at 37°C. Some plates contained fibronectin, HGF or TGF- β at the indicated

concentrations. The cells were then harvested and stained with acridine orange and ethidium bromide. The cells were counted under a fluorescence microscope.

Figure 7. Survival assay showing the effect of wortmannin on fibronectin and growth factor-induced SP1 cell survival. SP1 cells (20,000 per dish) were seeded in 1.5 ml RPMI 1640 with 1% FBS on agar coated plates (35 x 10mm) for 24 h at 37°C. The medium was supplemented with fibronectin (10 µg/ml), HGF (10 µg/ml) or TGF-β (0.5 ng/ml) as indicated. Wortmannin was added to some groups at a concentration of 100 µM. After 24 h the cells were then harvested and stained with acridine orange and ethidium bromide. The cells were counted under a fluorescence microscope. Live cells stained with a green nucleus and dead cells stained with a red nucleus. The experiments were repeated 4 times and the mean ± SD calculated.

Figure 8. Survival assay showing the effect of fibronectin and HGF-mediated survival on SP1 transfected with a dominant negative mutant of the p85 subunit (Δp85) of PI 3-kinase. C22 and C23 clones (20,000) were seeded in 1.5 ml RPMI 1640 with 1% FBS on agar coated plates (35 x 10mm) for 24 h at 37°C. Some plates contained fibronectin (10 µg/ml) or HGF (10 µg/ml) or FN + HGF. The cells were then harvested and stained with acridine orange and ethidium bromide. The cells were counted using a fluorescence microscope. Live cells stained with a green nucleus and dead cells stained with a red nucleus. The experiments were repeated 4 times and the mean ± SD calculated.

Figure 9. Western blot of PI 3-kinase phosphorylation. SP1 cells were pretraved for 24 h and incubated in 10 ml tubes in the presence of fibronectin (40µg/ml) or collagen (40µg/ml)

for 60 min at 37°C. The cells were lysed in 1% NP-40 lysis buffer and PI 3-kinase was immunoprecipitated with anti-PI 3-kinase rabbit IgG. The immunoprecipitates were washed and resolved by 8.0% SDS-PAGE under reducing conditions. The proteins were transferred to nitrocellulose and blotted with anti-phosphotyrosine antibody and visualized by ECL.

Figure 10. PI 3-kinase assay under anchorage-independent conditions in the presence of fibronectin and HGF. SP1 cells were maintained anchorage-independently for 1 h in the absence or presence of HGF or FN. The Cells were lysed in 1% NP40 lysis buffer and PI 3-kinase immunoprecipitated with anti-phosphotyrosine antibody. The immunoprecipitates were washed and incubated with the substrate phosphoinositol and 15 μ Ci gamma 32 P. After 20 min the reaction was stopped with 6M HCl and the lipids were extracted with chloroform/methanol and resolved by TLC. The plate was autoradiographed and the radioactive area corresponding to the radiolabelled phosphoinositol 3-phosphate (PI 3-P) was removed and measured in a scintillation counter.

Figure 1

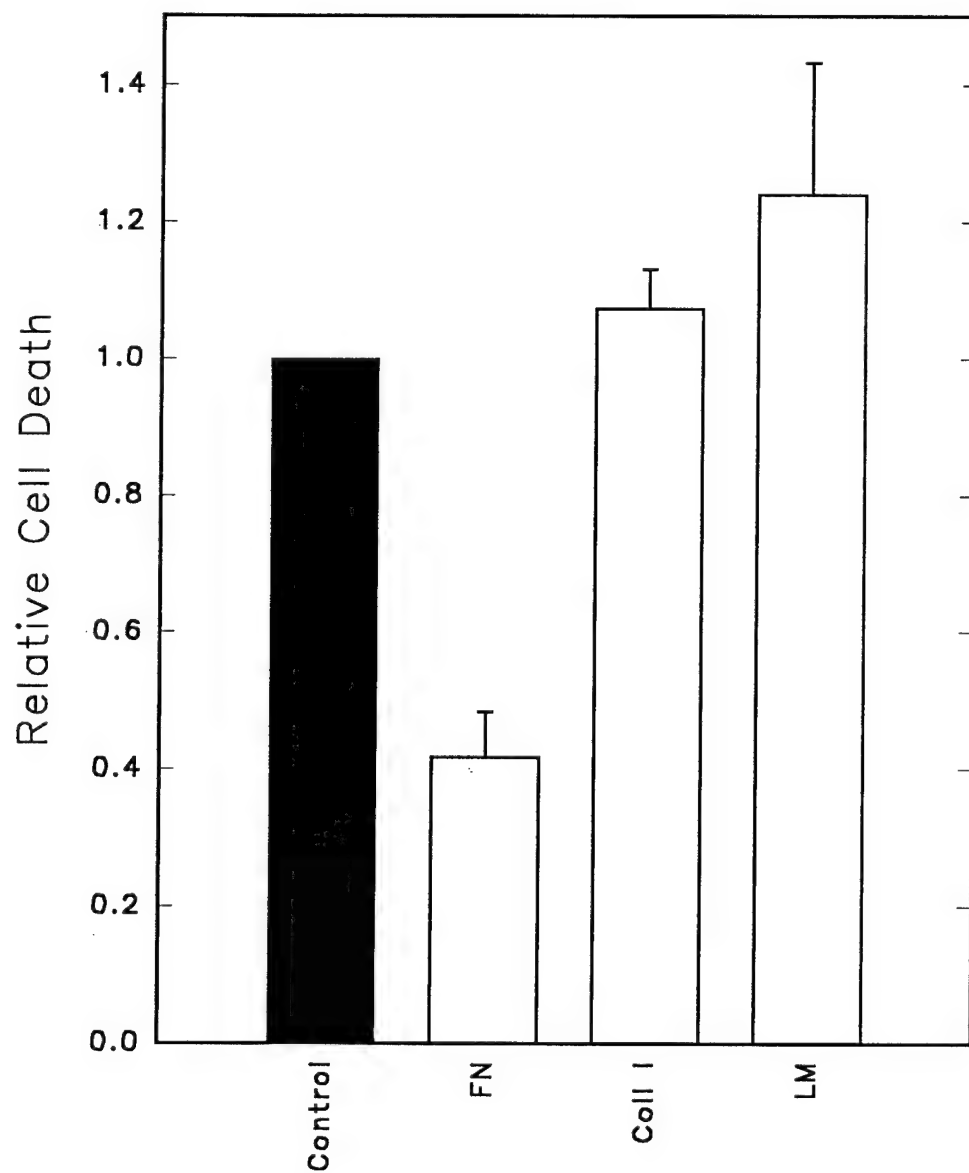


Figure 2

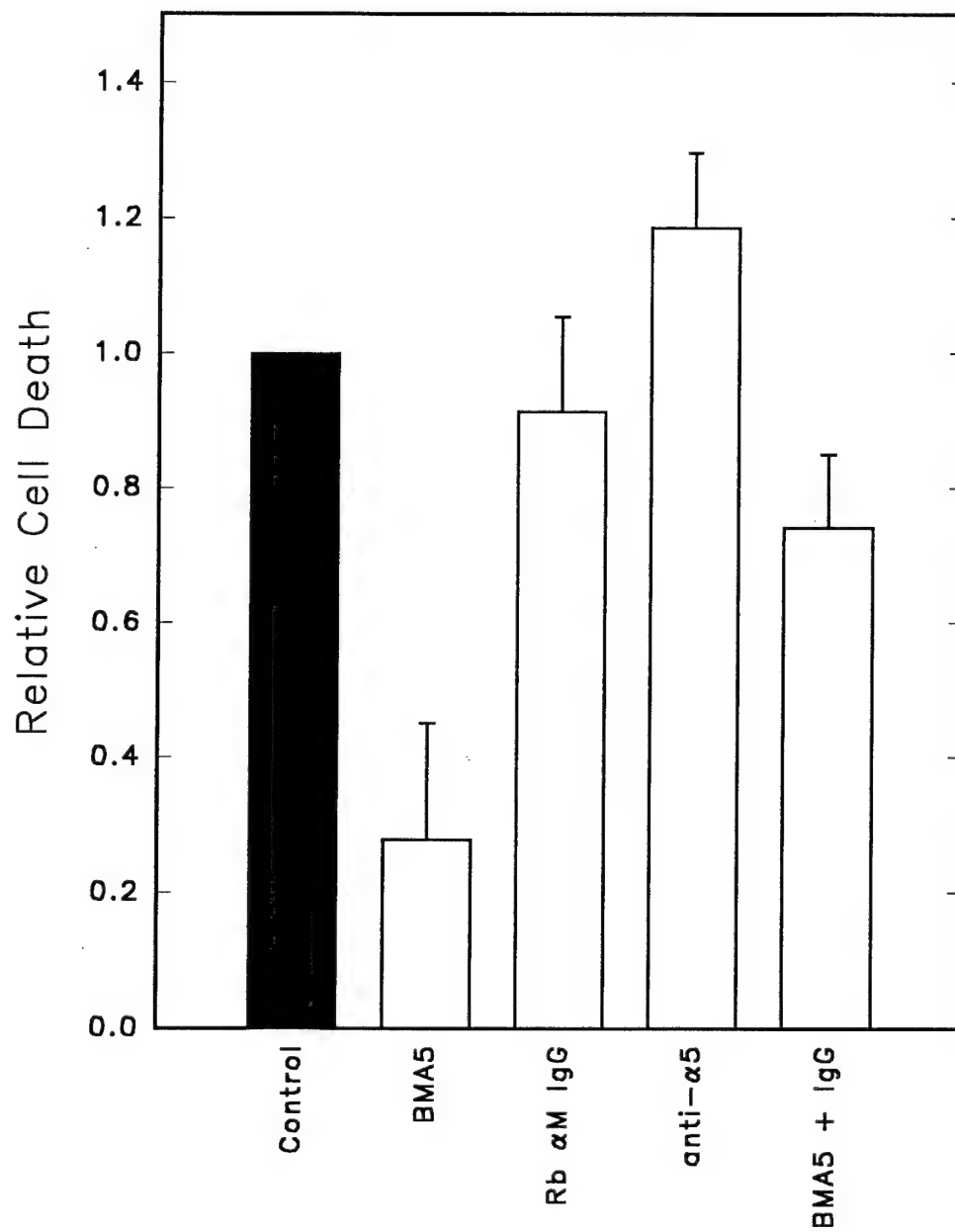
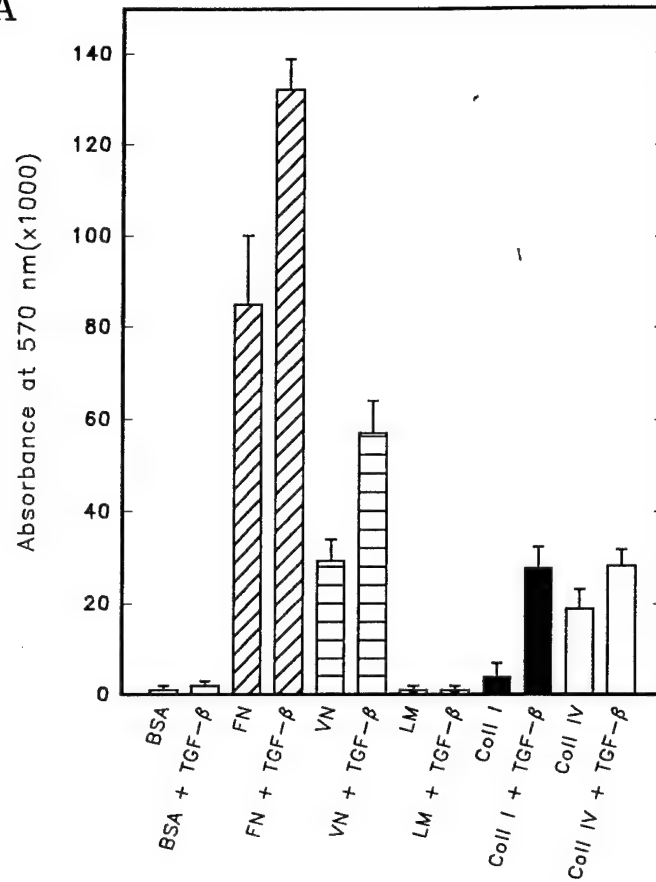


Figure 3

A



B

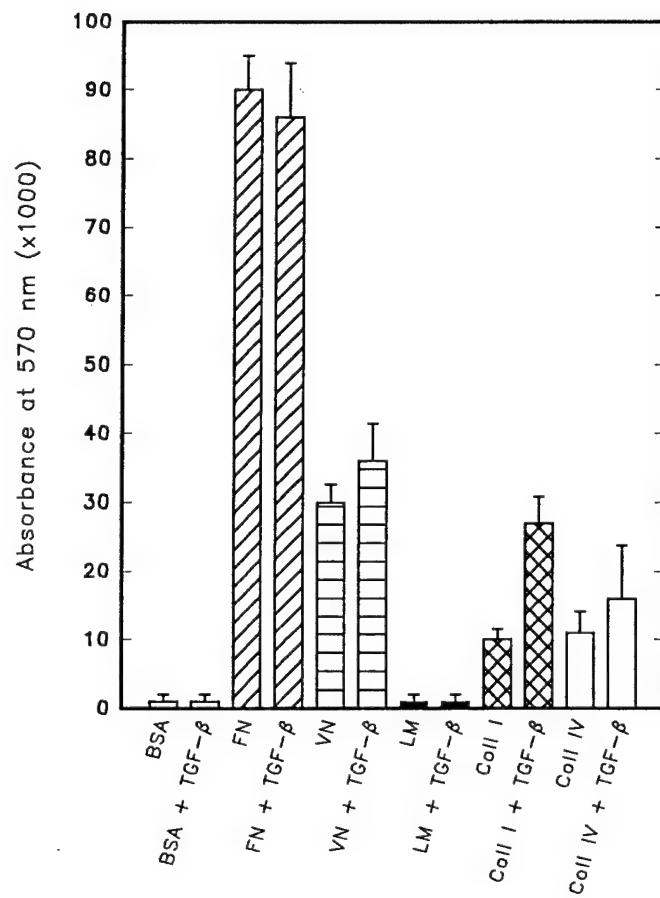


Figure 4

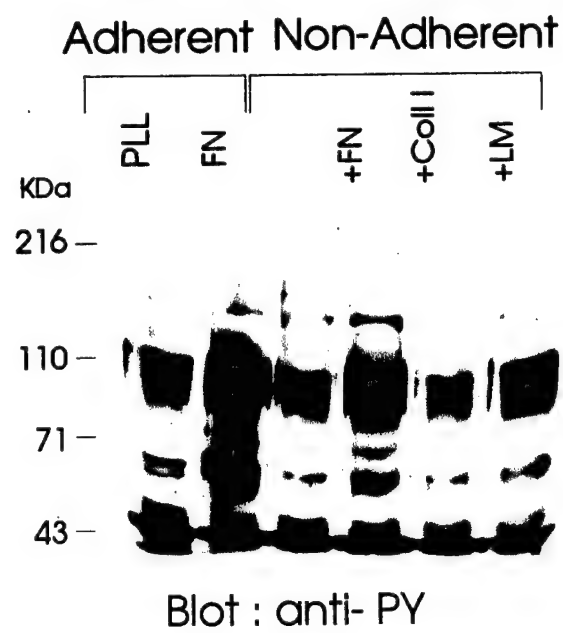


Figure 5

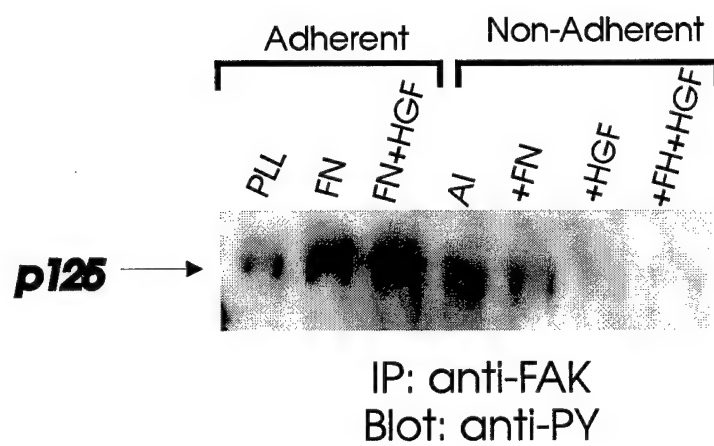


Figure 6

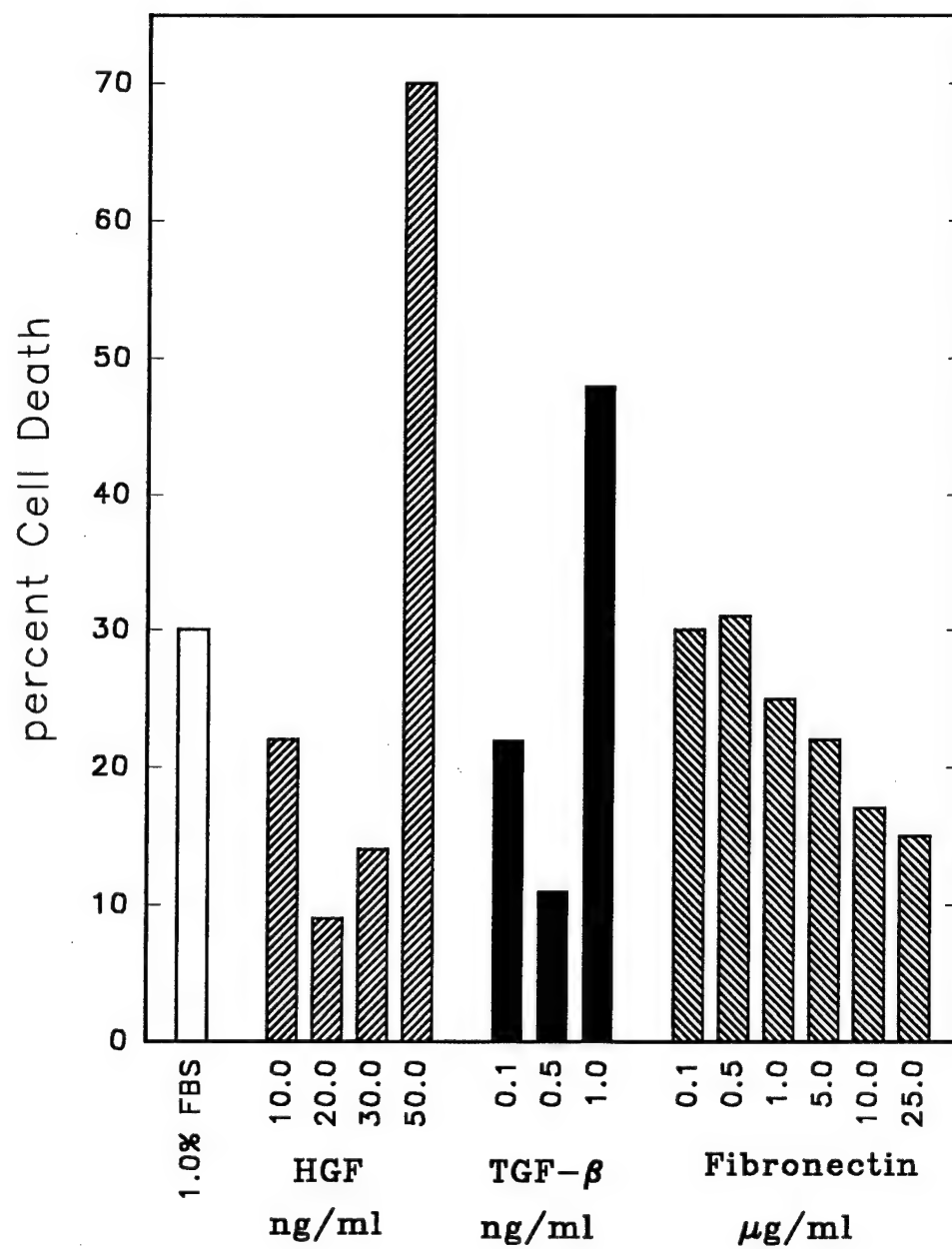


Figure 7

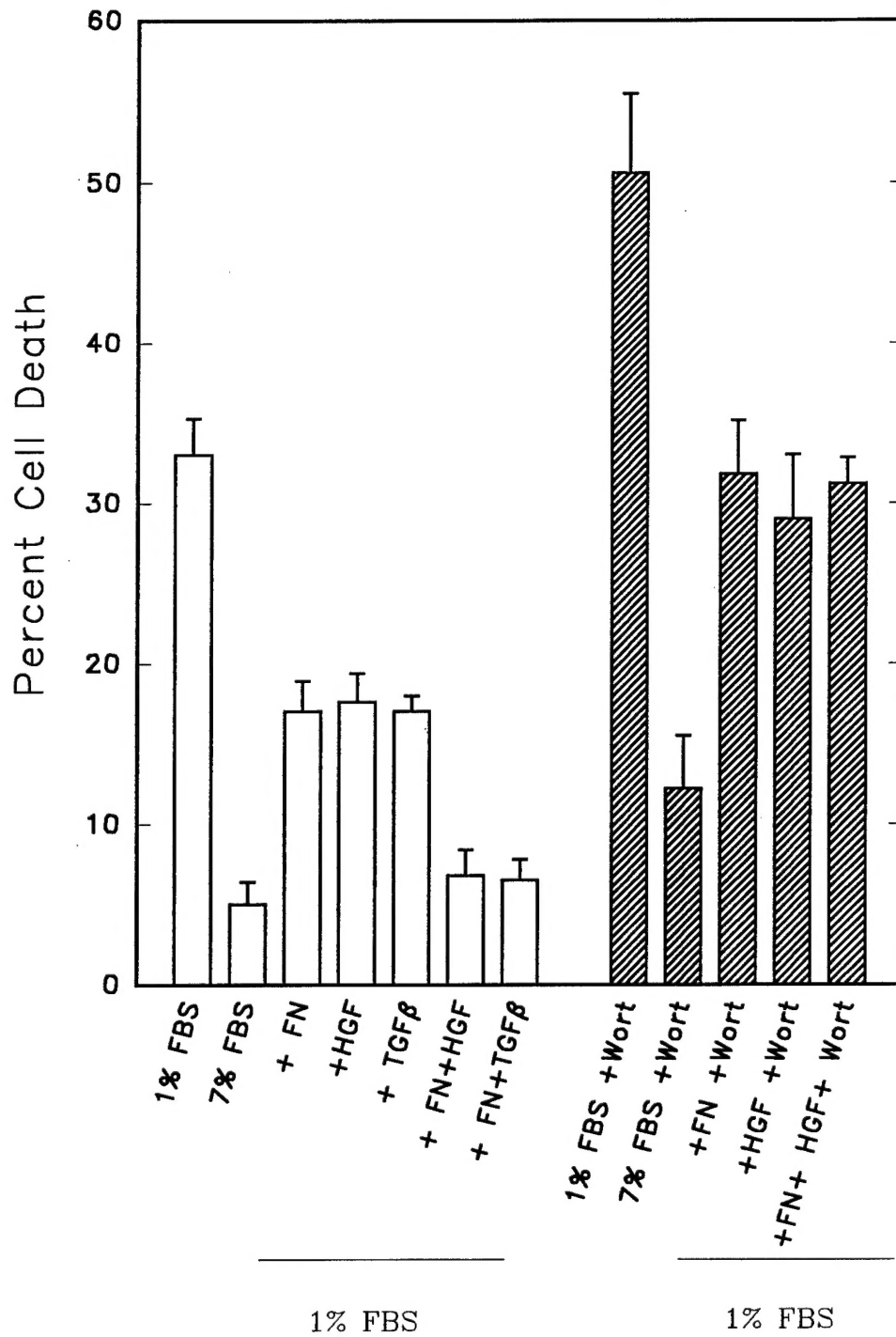


Figure 8

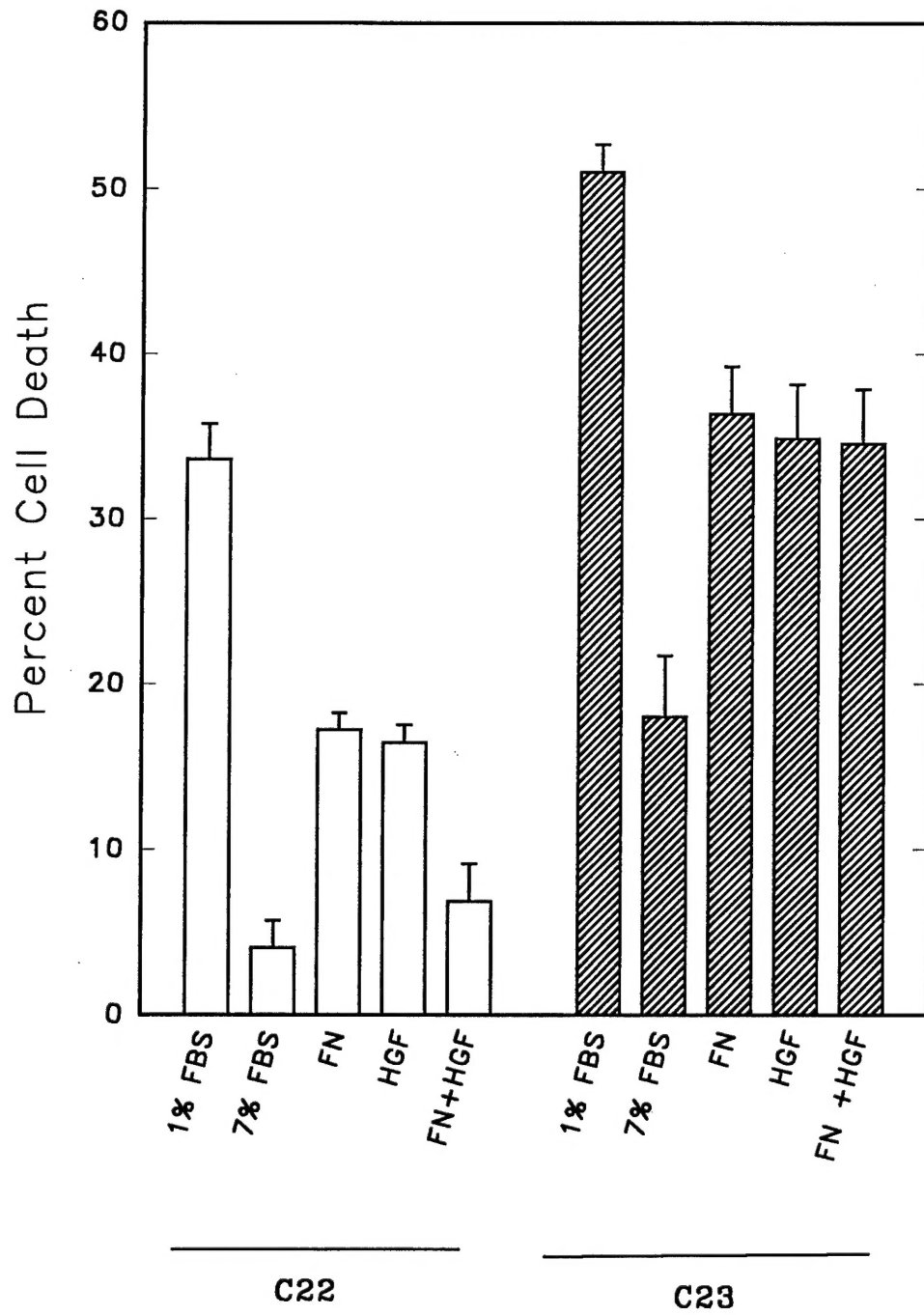
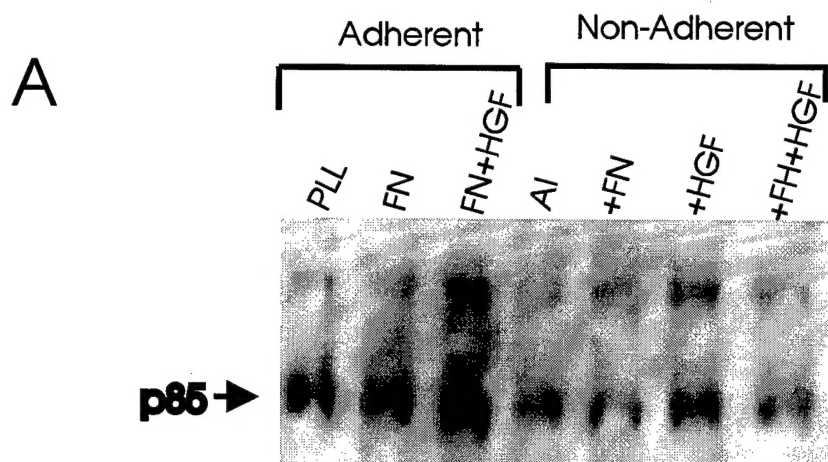
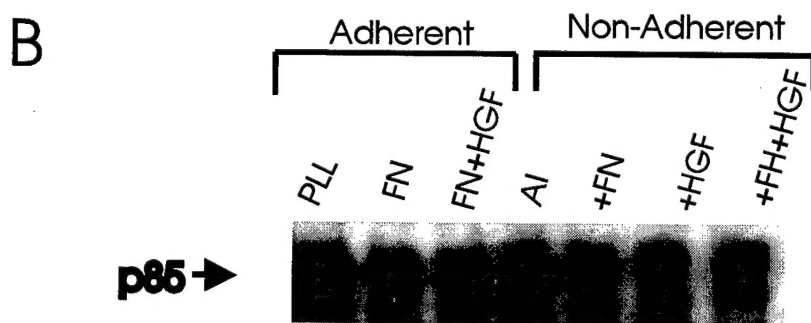


Figure 9



IP: anti-PI 3-kinase

Blot: anti-PY



IP: anti-PI 3-kinase
blot: anti-PI 3-kinase

Figure 10

